Docket No: 4765

Modified DNA-Polymerase from Carboxydothermus hydrogenoformans and its use for Coupled Reverse Transcription and Polymerase Chain Reaction

This application claims priority to European patent application No. 97.121151.1, filed December 2, 1997.

Background of the Invention

Field of the Invention

The present invention relates to a modified DNA-polymerase having reverse transcriptase activity and reduced 5'-3' exonuclease activity derived from a native polymerase which is obtainable from *Carboxydothermus hydrogenoformans*. Furthermore the invention relates to the field of molecular biology and provides methods for amplifying a DNA segment from an RNA template using an enzyme with reverse transcriptase activity (RT-PCR). In another aspect, the invention provides a kit for Coupled High Temperature Reverse Transcription and Polymerase Chain Reaction.

Description of Related Art

Heat stable DNA polymerases (EC 2.7.7.7. DNA nucleotidyltransferase, DNA-directed) have been isolated from numerous thermophilic organisms (for example: Kaledin et al. (1980), *Biokhimiya* **45**, 644-651; Kaledin et al. (1981) *Biokhimiya* **46**, 1576-1584; Kaledin et al. (1982) *Biokhimiya* **47**, 1785-1791; Ruttimann et al. (1985) *Eur. J. Biochem.* **149**, 41-46; Neuner et al. (1990) *Arch. Microbiol.* **153**, 205-207). For some organisms, the polymerase gene has been cloned and expressed (Lawyer et al. (1989) *J. Biol. Chem.* **264**, 6427-6437; Engelke et al. (1990) *Anal. Biochem.* **191**, 396-400; Lundberg et al. (1991) *Gene* **108**, 1-6; Perler et al. (1992) *Proc. Natl. Acad. Sci.* USA **89**, 5577-5581).

Thermophilic DNA polymerases are increasingly becoming important tools for use in molecular biology and there is growing interest in finding new polymerases which have more suitable properties and activities for use in diagnostic detection of RNA and DNA, gene cloning and DNA sequencing. At present, the thermophilic DNA polymerases mostly used for these purposes are from Thermus species like Taq polymerase from *T. aquaticus* (Brock et al. (1969) *J. Bacteriol.* **98**, 289-297).

The term "reverse transcriptase" describes a class of polymerases characterized as RNA-dependent DNA-polymerases. All known reverse transcriptases require a primer to synthesize a DNA-transcript from an RNA template. Historically, reverse transcriptase has been used primarily to transcribe mRNA into cDNA which can then be cloned into a vector for further manipulation.

Reverse transcription is commonly performed with viral reverse transcriptases like the enzymes isolated from *Avian myeloblastosis* virus or *Moloney murine leukemia* virus. Both enzymes mentioned are active in the presence of magnesium ions but have the disadvantages to possess RNase H-activity, which destroys the template RNA during the reverse transcription reaction and have a temperature optimum at 42°C or 37 °C, respectively. Avian myoblastosis virus (AMV) reverse transcriptase was the first widely used RNA-dependent DNA-polymerase (Verma (1977) *Biochem. Biophys. Acta* **473**, 1). The enzyme has 5'-3' RNA-directed DNA polymerase activity, 5'-3' DNA directed DNA polymerase activity, and RNaseH activity. RNaseH is a processive 5'-3' ribonuclease specific for the RNA strand of RNA-DNA hybrids (Perbal (1984), A Practical Guide to Molecular Cloning, Wiley & Sons New York). Errors in transcription cannot be corrected because known viral reverse transcriptases lack the 3'-5' exonuclease activity necessary for proofreading (Saunders and Saunders (1987) Microbial Genetics Applied to Biotechnology, Croom Helm, London). A detailed study of the activity of AMV reverse transcriptase and its associated RNaseH activity has been presented by Berger et al., (1983) *Biochemistry* **22**, 2365-2372.

DNA polymerases isolated from mesophilic microorganisms such as *E. coli* have been extensively characterized (see, for example, Bessmann et al. (1957) *J. Biol. Chem.* **233**, 171-177 and Buttin and Kornberg (1966) *J. Biol. Chem.* **241**, 5419-5427). *E. coli* DNA polymerase I (Pol I) is useful for a number of applications including: nick-translation reactions, DNA sequencing, in vitro mutagenesis, second strand cDNA synthesis, polymerase chain reactions (PCR), and blunt end formation for linker ligation (Maniatis et al., (1982) Molecular Cloning: A Laboratory Manual Cold Spring Harbor, New York).

Several laboratories have shown that some polymerases are capable of *in vitro* reverse transcription of RNA (Karkas (1973) *Proc. Nat. Acad. Sci.* USA **70**, 3834-3838; Gulati et al. (1974) *Proc. Nat. Acad. Sci.* USA **71**, 1035-1039; and Wittig and Wittig, (1978) *Nuc. Acids Res.* **5**, 1165-1178). Gulati et al. found that *E. coli* Pol I could be used to transcribe Qß viral RNA using oligo$(dT)_{10}$ as a primer. Wittig and Wittig have shown that *E.coli* Pol I can be used to reverse transcribe tRNA that has been enzymatically elongated with oligo(dA). However, as Gulati et al. demonstrated, the amount of enzyme required and the small size of cDNA product suggest that the reverse transcriptase activity of *E. coli* Pol I has little practical value.

Alternative methods are described using the reverse transcriptase activity of DNA polymerases of thermophilic organisms which are active at higher temperatures. Reverse transcription at higher temperatures is of advantage to overcome secondary structures of the RNA template which could result in premature termination of products. Thermostable DNA polymerases with reverse transcriptase activities are commonly isolated from Thermus species. These DNA polymerases however, show reverse transcriptase activity only in the presence of manganese ions. These reaction conditions are suboptimal, because in the presence of manganese ions the polymerase copies the template RNA with low fidelity.

Another feature of the commonly used reverse transcriptases is that they do not contain 3′-5′exonuclease activity. Therefore, misincorporated nucleotides cannot be removed and thus the cDNA copies from the template RNA may contain a significant degree of mutations.

One of the known DNA polymerases having high reverse transcriptase activity is obtainable from *Thermus thermophilus* (Tth polymerase) (WO 91/ 09944). Tth polymerase, as well as Taq polymerase, lacks 3' to 5' exonucleolytic proofreading activity. This 3' to 5' exonuclease activity is generally considered to be desirable because it allows removal of misincorporated or unmatched bases in the newly synthesized nucleic acid sequences. Another thermophilic pol I-type DNA polymerase isolated from *Thermotoga maritima* (Tma pol) has 3' to 5' exonuclease activity. U.S. patent 5,624,833 provides means for isolating and producing Tma polymerase. However, both DNA polymerases, Tth as well as Tma polymerase, show reverse transcriptase activity only in the presence of manganese ions.

The DNA polymerase of *Carboxydothermus hydrogenoformans* shows reverse transcription activity in the presence of magnesium ions and in the substantial absence of manganese ions and can be used to reverse transcribe RNA, to detect and amplify (in combination with a thermostable DNA polymerase like Taq) specific sequences of RNA. Using DNA polymerase of *Carboxydothermus hydrogenoformans* polymerase a high specificity of transcription is observed with short incubation times. A high specificity is observed using e.g. 5 min of incubation time and 33 units of DNA polymerase protein. With longer incubation times also with lower amounts of *Carboxydothermus hydrogenoformans* polymerase specific products can be obtained. However an unspecific smear of products is occurring. These unspecific products might be caused by the 5´-3´exonuclease activity of the polymerase which enables the enzyme to cleave the template at secondary structures ("RNaseH"-activity) and to create additional primers which can be elongated by the DNA polymerase activity. The thermostable DNA polymerase from *Carboxydothermus hydrogenoformans* has been identified and cloned and is described in the copending European application with the Application No. 96115873.0, filed October 03, 1996, and incorporated herein by reference.

In summary, reverse transcriptases as MoMULV-RT or AMV-RT perform reverse transcription in the presence of magnesium-ions. However, these enzymes act at temperatures between 37°C and 55°C. Reverse transcription at higher temperatures would be desirable because secondary

structures can be overcome in the template in order to avoid premature termination of the reaction and to assure the production of cDNA without deletions. Other enzymes e.g. DNA polymerase obtainable from *Thermus spec.* act as reverse transcriptase at temperatures up to 70°C in the presence of manganese ions. These reaction conditions are suboptimal, because in the presence of manganese ions the polymerase copies the template RNA with low fidelity and the RNA strand will be degraded. Degradation of the RNA strand occurs faster in the presence of manganese ions as in the presence of magnesium ions. Therefore, if manganese ions are present complexation of the manganese ions (e.g. with EDTA) is required after cDNA synthesis in order to obtain a higher fidelity during cDNA amplification in the subsequent PCR reaction.

Therefore, it is desirable to develop a reverse transcriptase

- which acts at higher temperatures to overcome secondary structures in the template to avoid premature termination of the reaction and to assure the production of cDNA without deletions
- which is active in the presence of magnesium ions in order to prepare cDNA from RNA templates with higher fidelity and
- which has 3′-5′-exonuclease in order to remove misincorporated nucleotides before continuation of DNA synthesis and to produce products with low mutation frequency
- which has a high specificity and produces exclusively or predominantly RT-PCR products derived from specific primer binding.

Summary of the Invention

The present invention addresses these needs and provides a DNA polymerase mutant active at higher temperatures which has reverse transcriptase activity in the presence of magnesium ions and which has 3′-5′exonuclease activity and reduced or no 5'-3' exonuclease activity.

It is an object of this invention to provide a polymerase enzyme (EC 2.7.7.7.), characterized in that it has reverse transcriptase activity in the presence of magnesium ions as well as in the presence of manganese ions. In a further aspect the invention comprises a DNA polymerase having 3′-5′-exonuclease activity and reduced 5'-3' exonuclease activity. The enzyme according to the invention can be obtained from a polymerase obtainable from *Carboxydothermus hydrogenoformans* (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, DSM No. 8979). In a further aspect the invention is directed to a DNA polymerase with reduced 5'-3' exonuclease activity having reverse transcriptase activity in the presence of magnesiums ions and in the substantial absence of manganese ions. In a further aspect the invention comprises a DNA polymerase having a molecular mass of about 64 to 71 kDa as determined by SDS PAGE analysis. The mutant polymerase enzyme with reduced 5'-3' exonuclease activity derived from a polymerase obtainable from *Carboxidothermus hydrogenoformans* is called hereinafter Δ Chy Polymerase.

In a further aspect the invention comprises a recombinant DNA sequence that encodes DNA polymerase activity of the Δ Chy Polymerase. In a related aspect, the DNA sequence is depicted as SEQ ID No. 10 (Figure 1). In a second related aspect the invention comprises a recombinant DNA sequence that encodes essentially amino acid residues 1 to 607 (SEQ ID No. 11, Figure 1). In a further aspect the invention comprises a recombinant DNA plasmid that comprises the DNA sequence of the invention inserted into plasmid vectors and which can be used to drive the expression of the Δ Chy DNA polymerase in a host cell transformed with the plasmid. In a further aspect the invention includes a recombinant strain comprising the vector pDS56 carrying the Δ Chy DNA polymerase gene and designated $p\Delta_{2\text{-}225}AR_4$. The E.coli strain XL1 carrying the plasmid $p\Delta_{2\text{-}225}AR_4$ was deposited on the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascherorder Weg 1b, D-38124 Braunschweig DSM No. 11854 (BMTU 7307) is designated *E.coli* GA1.